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Insulin and heparin suppress superoxide production in diabetic rat glomeruli stimulated with low-density lipoprotein

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Insulin and heparin suppress superoxide production in diabetic rat glomeruli stimulated with low-density lipoprotein. Patients with diabetic nephropathy frequently show increased levels of circulating low-density lipoprotein (LDL) and oxidized LDL, which have been reported to be related to the generation of oxygen-free radicals. In the present study, we evaluated the effects of insulin and heparin on the superoxide production of glomeruli, which were isolated from rats with streptozotocin-induced diabetes for one week, one month, and three months, respectively, and the glomeruli were stimulated with native and oxidized LDL. LDL was isolated from normal subjects with normolipidemia, and the superoxide was measured by using a spectrophotometer. The results demonstrated that the poorly controlled diabetic rat glomeruli showed a significantly higher production of superoxide than normal glomeruli under basal status and after stimulation, and this production increased further with the progression of diabetes. Insulin suppressed both the basal and stimulated production of superoxide in diabetic glomeruli, but not in normal glomeruli. Heparin suppressed superoxide production of diabetic glomeruli stimulated by either native or oxidized LDL, and it also partly suppressed superoxide production of normal glomeruli stimulated by oxidized LDL. Our results suggest that glomerular injury in diabetics with hyperlipidemia may be mediated through enhanced generation of oxygen-free radicals, which can be partially attenuated by insulin and heparin.

Glomerulosclerosis is a major morphological change in patients with diabetic nephropathy. Recent studies indicate that glomerulosclerosis and atherosclerosis are based on similar pathophysiologic mechanisms [1], and both are closely related with hyperlipidemia and the production of oxygen-free radicals (OFRs). Many studies have suggested that oxidation of low-density lipoprotein (LDL) particles is a critical event in the development of atherosclerosis, and the presence of oxidized LDL correlates with the progression of atherosclerosis [2]. This "oxidative-modification hypothesis" of atherosclerosis may be equally important in the development of glomerulosclerosis in diabetic nephropathy because all of the elements for atherosclerosis are present in the renal glomerulus.

Patients with diabetic nephropathy show increased levels of OFR in both plasma and renal tissues, and an increased level of LDL is also observed in patients with non–insulin-dependent diabetes mellitus [3]. This atherogenic lipoprotein profile becomes more important when diabetic nephropathy is present, and effective normalization of hypercholesterolemia with lovastatin, a HMG-CoA reductase inhibitor, may retard the progression of diabetic nephropathy [4]. LDL appears to be more prone to oxidation in diabetes [5], and circulating levels of oxidized LDL have been found to be elevated in diabetic patients [6]. In the present study, we measured the superoxide production of freshly isolated diabetic rat glomeruli under basal conditions or stimulated with native and oxidized LDL, and we observed whether insulin and heparin had some protective effects on this oxidative damage.

METHODS

Materials

Male Wistar rats with body weights of between 200 and 250 g were used for the study. Rats were injected intraperitoneally with 55 mg/kg of streptozotocin (Sigma Chemical, St. Louis, MO, USA) and were used for experimentation after confirming the diagnosis of diabetes mellitus. Rats were classified into three groups, each containing 10 rats. Group I was sacrificed one week after inducing diabetes (DM1W), group II was sacrificed after one month (DM1M), and group III after three months (DM3M). All three groups had the same number of rats sacrificed at the same time to serve as normal controls. Groups II and III were controlled with insulin (heat-treated bovine ultralente insulin; Novo Nordisk, Copenhagen, Denmark) every day to maintain a poorly controlled diabetic state so that the deleterious effects may be enhanced during the short study period. Plasma glucose levels were regularly checked and maintained above

Key words: diabetic nephropathy, glomerular injury, hyperlipidemia, oxygen free radicals, glomerulosclerosis, atherosclerosis

Table 1. Effects of insulin (0.3 mU/mL) on the superoxide production (nmol/mg glomerular protein) of normal and diabetic glomeruli isolated from rats with different duration of diabetes.

Rat group	No stimulation		LDL stimulation		Ox-LDL stimulation	
	Insulin(−)	Insulin(+)	Insulin(−)	Insulin(+)	Insulin(−)	Insulin(+)
	(A)	(B)	(C)	(D)	(E)	(F)
DM1W	1.92 ± 0.31	1.49 ± 0.21[b]	3.11 ± 0.41	2.02 ± 0.37[c]	6.91 ± 0.69	3.88 ± 0.60[d]
NC1W	1.51 ± 0.09	1.57 ± 0.18	1.71 ± 0.30	1.78 ± 0.31	3.12 ± 0.34	2.99 ± 0.54
DM1M	2.21 ± 0.49	1.60 ± 0.27[a]	3.39 ± 0.49	2.38 ± 0.25[c]	6.33 ± 0.47	4.14 ± 0.48[d]
NC1M	1.45 ± 0.13	1.51 ± 0.21	1.84 ± 0.37	1.89 ± 0.38	3.01 ± 0.29	3.13 ± 0.39
DM3M	2.67 ± 0.32	1.68 ± 0.15[a]	3.92 ± 0.37	2.34 ± 0.27[c]	6.50 ± 0.41	4.57 ± 0.45[d]
NC3M	1.57 ± 0.14	1.49 ± 0.14	1.79 ± 0.34	1.91 ± 0.29	3.15 ± 0.25	3.02 ± 0.42

The glomeruli were either unstimulated or stimulated with low-density lipoprotein (LDL) or oxidized-LDL in the presence or absence of insulin.
Definitions are: DM1W, DM for 1 week; NC1W, normal controls for DM1W; DM1M, DM for 1 month; NC1M, normal controls for DM1M; DM3M, DM for 3 months; NC3M, normal controls for DM3M, Insulin(−), no insulin added; Insulin(+), preincubated with insulin.
[a] $P < 0.01$, [b] $P < 0.05$, compared column B to column A of the same row
[c] $P < 0.01$ compared column D to column C of the same row
[d] $P < 0.01$ compared column F to column E of the same row

450 mg/dL (498 to 7711 mg/dL, range 435 to 609 mg/dL, $N = 341$). All rats were allowed food and water ad libitum.

Experimental protocol

The freshly isolated diabetic or normal rat glomeruli were stimulated with either native or oxidized LDL at a concentration of 50 μg/dL for six hours. The amount of superoxide production was measured at basal status and after stimulation, and the effects of pretreatment with insulin and heparin were evaluated. To test the effect of insulin in vitro, the diabetic or normal glomeruli were initially incubated with insulin (0.3 mU/mL; Sigma Chemical Co.) at 4°C for 90 minutes and then incubated for 30 minutes at 37°C. It should be emphasized that the initial exposure of diabetic glomeruli to insulin at low temperature was required for expression of its effects [7]. In another experiment, the diabetic and normal glomeruli were also preincubated with heparin (100 μg/mL; Sigma Chemical Co.) at 37°C for two hours before stimulation with native or oxidized LDL.

Preparation of glomeruli

Diabetic and normal rats were anesthetized with pentobarbital, and both kidneys were collected. The glomeruli were obtained by sieving the renal cortex, and we could obtain glomeruli at 95% purity. The viability of the isolated glomeruli was greater than 96%. The collected glomeruli were then incubated in RPMI 1640 medium supplemented with 20% fetal calf serum and antibiotics and were used immediately for experiments. By the end of each experiment, glomeruli were homogenized, and the protein content was measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

Isolation of LDL and preparation of oxidized LDL

Human plasma LDL was prepared from normolipidemic fasting volunteers according to a method described previously [8]. Briefly, after the addition of 0.02% ethylenediaminetetraacetic acid (EDTA) and 0.05% sodium azide to the plasma, LDL (d = 1.019 to 1.064 g/mL) was prepared by ultracentrifuge separation at 15°C. LDL was transferred into a dialysis bag and dialyzed for 24 hours at 4°C against Dulbecco's phosphate-buffered saline (PBS) containing 0.02% EDTA. To oxidize LDL, EDTA was removed by dialysis, and the LDL was incubated in Dulbecco's PBS containing 10 μmol/L $CuSO_4$ for 24 hours at 37°C.

Measurement of superoxide

Superoxide anion production by glomeruli was determined by measuring superoxide dismutase (SOD) inhibitable reduction of cytochrome C, as previously described [9]. Briefly, the glomeruli (10^4/mL) in PBS containing Ca^{2+} (1 mmol/L) and cytochrome C (80 μmol/L) with or without SOD (500 μg/mL) were preincubated for 10 minutes in a 37°C water bath. The glomeruli were then stimulated with LDL or oxidized LDL at 37°C for six hours. The absorbance of the supernatant was then measured by using a spectrophotometer at 550 nm.

Statistics

All results are expressed as mean values ± SEM. One-way analysis of variance (ANOVA) with posteriori tests between groups was used to compare the differences.

RESULTS

Incubation of LDL with $CuSO_4$ resulted in the formation of 18.3 ± 0.3 nmol of thiobarbituric acid reactive substance per milligram of LDL. Oxidation of LDL was also characterized by the increased negative charge, as indicated by the increased electrophoretic migration on agarose gel. The basal superoxide production was significantly higher in diabetic than in normal glomeruli (Table 1). The superoxide production was also signifi-

Table 2. Effects of heparin (100 μg/ml) on the superoxide production (nmol/mg glomerular protein) of normal and diabetic glomeruli isolated from rats with different duration of diabetes

Rat group	No stimulation		LDL stimulation		Ox-LDL stimulation	
	Heparin(−)	Heparin(+)	Heparin(−)	Heparin(+)	Heparin(−)	Heparin(+)
	(A)	(B)	(C)	(D)	(E)	(F)
DM1W	1.92 ± 0.31	1.67 ± 0.24[b]	3.11 ± 0.41	2.27 ± 0.39[c]	6.91 ± 0.69	4.25 ± 0.71[d]
NC1W	1.51 ± 0.09	1.45 ± 0.17	1.71 ± 0.30	1.67 ± 0.27	3.12 ± 0.34	2.67 ± 0.49[e]
DM1M	2.21 ± 0.49	1.72 ± 0.32[a]	3.39 ± 0.49	2.50 ± 0.31[c]	6.33 ± 0.47	4.77 ± 0.64[d]
NC1M	1.45 ± 0.13	1.52 ± 0.19	1.84 ± 0.37	1.59 ± 0.41	3.01 ± 0.29	2.87 ± 0.31
DM3M	2.67 ± 0.32	1.90 ± 0.27[a]	3.92 ± 0.37	2.78 ± 0.34[c]	6.50 ± 0.41	5.09 ± 0.38[d]
NC3M	1.57 ± 0.14	1.39 ± 0.22	1.79 ± 0.34	1.68 ± 0.29	3.15 ± 0.25	2.51 ± 0.34[e]

The glomeruli were either unstimulated or stimulated with LDL or oxidized-LDL in the presence or absence of heparin.

Groups are defined as: DM1W, DM for 1 week; NC1W, normal controls for DM1W; DM1M, DM for 1 month; NC1M, normal controls for DM1M; DM3M, DM for 3 months; NC3M, normal controls for DM3M; Heparin(−), no heparin added; heparin(+), preincubated with heparin.

[a] $P < 0.01$, [b] $P < 0.05$ compared column B to column A of the same row

[c] $P < 0.01$ compared column D to column C of the same row

[d] $P < 0.01$, [e] $P < 0.05$ compared column F to column E of the same row

cantly higher in the glomeruli isolated from DM3M rats than in those isolated from DM1W and DM1M rats (both $P < 0.05$). The superoxide production of the diabetic glomeruli increased significantly after stimulation with LDL or oxidized LDL (Table 1). The superoxide production of diabetic glomeruli after stimulation with oxidized LDL was significantly higher than that of those stimulated with native LDL ($P < 0.001$ for all three groups).

Insulin significantly suppressed basal production of superoxide by diabetic glomeruli ($P < 0.05$ for DM1W, $P < 0.01$ for DM1M and DM3M; Table 1), while basal production of superoxide by normal glomeruli was not suppressed. Insulin also significantly suppressed the superoxide production of diabetic glomeruli stimulated by both native and oxidized LDL ($P < 0.01$ for all). The superoxide production of normal glomeruli, which were also enhanced after oxidized LDL stimulation, was not suppressed by insulin.

Heparin significantly suppressed basal production of superoxide by diabetic glomeruli ($P < 0.05$ for DM1W, $P < 0.01$ for DM1M and DM3M; Table 2). Basal production of superoxide by normal glomeruli was not suppressed by heparin. Heparin also significantly suppressed the superoxide production of diabetic glomeruli stimulated by native and oxidized LDL ($P < 0.01$ for all). While it had no effect on the LDL-stimulated superoxide production of normal glomeruli, heparin partially suppressed the oxidized LDL-stimulated superoxide production of normal glomeruli.

DISCUSSION

The current study demonstrates that both insulin and heparin suppress superoxide production by diabetic glomeruli stimulated by LDL and oxidized LDL. There is considerable evidence suggesting that the generation of OFR is increased in diabetes, and the OFR is closely related with chronic complications of diabetes, including diabetic nephropathy. Excessive generation of superoxide anion by diabetic glomeruli, as in our study, may play an important role in the oxidative modification of LDL. A recent study also demonstrated that the increased lipid peroxidation of LDL in vivo in diabetic subjects is the result of increased generation of OFR [10]. Although enhanced localization of oxidized LDL in diabetic kidneys has not been shown, a recent study demonstrated a tenfold increase in glomerular localization of oxidized LDL as compared with LDL when both were injected into suprarenal aorta of normal rats [11]. Therefore, oxidized LDL may be more important than native LDL in mediating OFR generation and ultimate glomerular injuries in diabetic kidneys.

Glomerulosclerosis is characterized by increased extracellular matrix and cell deletion. A recent study indicated that both native and oxidized LDL modulate mesangial cell apoptosis [12]. The mechanism for apoptosis, however, is largely caused by the generation of superoxide and OFR in general. Therefore, our results that oxidized LDL enhanced superoxide production by diabetic glomeruli may be an important mechanism by which LDLs induce diabetic glomerulosclerosis.

We have also observed the effect of insulin on the superoxide production of glomeruli in vitro. Although insulin treatment of diabetic rats has been shown to suppress the OFR generation [13], such in vivo studies cannot possibly separate the direct effects of insulin on glomeruli from multiple other insulin actions, including a reduction of blood sugar. In our in vitro study, the exposure of glomeruli to insulin decreased superoxide generation by diabetic glomeruli but not normal glomeruli, indicating that insulin deficiency plays an important role in the overproduction of superoxide in diabetic glomeruli. Previous studies have provided evidence for the presence of insulin receptors in isolated glomeruli [14] and thus indicate a basis for direct action of insulin on diabetic glomeruli in vitro. The discrepancy between the

effect of insulin on diabetic and normal glomeruli may be due to the fact that there is an increased insulin receptor number and/or increased receptor affinity in insulin-deficient rats.

Recent studies demonstrated that heparin prevents albuminuria and reverses the mesangial expansion occurring in diabetic rats [15]. The mechanisms by which heparin suppresses albuminuria are not clear. Our study demonstrates that the effect may partly be due to its suppression on the superoxide production of diabetic glomeruli. Heparin has been shown to inhibit selectively the protein kinase C-dependent pathway of signal transduction, which is also involved in the generation of OFR. Heparin has been isolated from mesangial cells and can exert a significant antimitogenic effect on rat mesangial cells at a concentration as low as 0.1 μg/mL [16], and this is important because the concentration is close to the levels of heparin-like molecules encountered in vivo. In addition to its suppressive effect, heparin also decreases binding of both LDL and oxidized LDL to cultured rat mesangial cells [17].

In summary, we have demonstrated that diabetic rat glomeruli produce more superoxide anion than normal glomeruli under basal status and after stimulation with native and oxidized LDL. Both insulin and heparin suppress superoxide production by diabetic glomeruli, and therefore, both drugs may have some effects on the prevention of this diabetic complication.

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